THIN-LAYER CHROMATOGRAPHIC IN SITU ANALYSIS OF
INSECT ECDYSONES VIA FLUORESCENCE-QUENCHING

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ABSTRACT

The possibility of quantitating insect ecdysones in situ on thin-layer chromatographic plates was examined. Two approaches were evaluated: 1) the induction of ecdysone fluorescence by sulfuric acid treatments and 2) the fluorescence-quenching of fluorescent thin-layer chromatographic plates by ecdysones. The fluorescence-quenching method was found to be most suitable and had a linear response range from 0.5 to 3 µg for α-ecdysone and 20-hydroxyecdysone. Fluorescence-quenching and high pressure liquid chromatographic analyses obtained from extracts of α-ecdysone 20-hydroxylase incubations gave similar results. New data concerning the acid-induced fluorescence of ecdysones showed α-ecdysone to be twice as fluorescent as 20-hydroxyecdysone.

INTRODUCTION

Although a considerable body of information has been gathered on

insect ecdysones (1-3), data have only recently been published

concerning some of the enzymes responsible for the metabolism of

ecdysones (4-7). We have been expressly concerned with the in-depth

study of the α-ecdysone 20-hydroxylase from the midgut of the tobacco

hornworm, Manducta sexta (L.). This enzyme hydroxylates α-ecdysone at

the 20-position to give 20-hydroxyecdysone (Figure 1). Purification of

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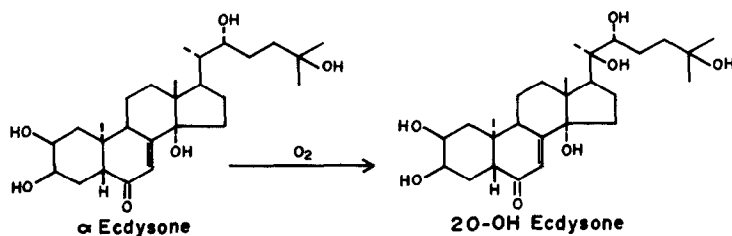the α-ecdysone 20-hydroxylase requires a more rapid analytical method

for α-ecdysone and 20-hydroxyecdysone.

Fig. 1.   Conversion of α-ecdysone to 20-hydroxyecdysone by α-ecdysone
20-hydroxylase.

The purification of α-ecdysone 20-hydroxylase or other ecdysone-

metabolizing enzymes requires testing of large numbers of column

fractions for specific activity.   However, analyses of α-ecdysone or

20-hydroxyecdysone can vary 10 to 25 min per sample depending on whether

gas liquid chromatography or high pressure liquid chromatography (HPLC)

are used (8-10).   In addition, extraction, sample preparation, and

column equilibration extend analysis time.

We have investigated the possibility of using analytical thin-layer

chromatography (TLC) as a more rapid alternative analytical method for

α-ecdysone and 20-hydroxyecdysone.   Two approaches were studied: (a)

the induction of ecdysone fluorescence by treatment with H_2SO_4; and (b)

the quenching of a fluorescent indicator impregnated in the TLC plates

by ecdysone.   The latter approach was more suitable and compared well

with HPLC analysis.

METHODS AND MATERIALS

Chemicals: α-Ecdysone and 20-hydroxyecdysone were purchased from Simes Pharmaceuticals, Milan, Italy, and Rohto Pharmaceutical Co., Osaka, Japan (11). α-Ecdysone was purified by column chromatography on silicic acid (12). Both α-ecdysone and 20-hydroxyecdysone were judged to be > 99% pure by HPLC analyses. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XV from bakers' yeast), and NADP were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade, and solvents were distilled from glass.

Preparation of Mitochondria: Midguts from prepupae of M. sexta were removed, cleaned by washing in buffered (10 mM tris-HCl, pH 7.4) 0.3 M sucrose solution, and then homogenized with a Teflon-glass homogenizer in 4 volumes of buffered sucrose solution. The homogenate was centrifuged at 1000g for 10 min, and the pellet was resuspended in 2 volumes of buffered sucrose and recentrifuged at 1000g for 10 min. Pooled supernatants were centrifuged a third time at 1000g for 10 min; then this supernatant was decanted and centrifuged again at 8000g for 20 min. The pellet was resuspended and centrifuged again at 8000g for 20 min; this step was repeated once more. The washed pellet was suspended in a half-volume of 50 mM potassium phosphate buffer (pH 7.7). Protein was assayed according to the method of Lowry et al. (13).

Enzyme Assays: Reaction mixtures each contained 2.8 μmol NADP, 76 μmol glucose-6-phosphate, 6.6 units glucose-6-phosphate dehydrogenase, 24 mg mitochondrial protein, and 50 mM potassium phosphate buffer to bring the total volume to 4 ml. These mixtures were combined in 125-ml boiling flasks and allowed to equilibrate for 3 min at 30°C in a Dubnoff shaker bath. The enzyme reaction was initiated by the addition of 0.43 μmol (200 μg) of α-ecdysone dissolved in 40 μl of methanol. The reactions were stopped by the addition of 15 ml methanol.

Extraction of Products: Extraction and fractionation of α-ecdysone and 20-hydroxyecdysone followed the procedures of Nigg et al. (5) and Kaplanis et al. (12).

HPLC Analysis: A modified procedure of Nigg et al. (8) was employed for HPLC analysis of α-ecdysone and 20-hydroxyecdysone. A Spectra-Physics HPLC equipped with a UV detector (254 nm) and three 1.0 m X 2 mm ID stainless steel columns packed with Corasil II connected in sequence were used in these analyses. The flow rate was set at ca. 1 ml per min, and an isocratic elution system of chloroform: 95% ethanol (7/1, v/v) was used. Samples were dissolved in 300 μl of methanol; 10 μl samples were injected into the HPLC for analysis.

TLC Analysis: Prepared TLC plates (20 X 20 cm; 0.25 mm thick) containing a fluorescent indicator (EM Labs, Elmsford, NY) were used. Samples in methanol solution were spotted on the plates with microsyringes. The TLC plates were developed with chloroform--95% ethanol (65/35, v/v) with wicks in a closed system. Developed plates were allowed to air dry and then either sprayed with a H_2SO_4--95% ethanol solution (3/1, v/v) (induced fluorescence) or quantitated

directly (fluorescence-quenching). The samples were read in a Kontes reflectance TLC scanner (Vineland, NJ) in the double-beam mode. Quantitation was achieved by cutting the peaks out and weighing them. The short-wave ultraviolet (UV) light source was used to excite the plates in the fluorescence-quenching experiments, and the long-wave UV light source was used in the acid-induced fluorescence studies.

In some cases, samples developed as described were scraped from the plates, and the silica gel was extracted three times with 10 ml of methanol. The methanolic extracts were evaporated with a stream of nitrogen and heated in a water bath. Ecdysone fluorescence was induced by addition of 3 ml H_2SO_4--95% ethanol (3/1, v/v) in a fashion similar to that described by Gilgan and Zinck (14); aliquots were placed in quartz cuvettes of 1-cm pathlength. The solution was stirred with a vortex mixer and allowed to incubate for 10 min. at ambient temperature before reading; the fluorescence was read with an AMINCO ratio spectrophotofluorometer. The excitation and emission wavelengths were fixed at 385 and 440 nm, respectively; the slit arrangement was 1.0, 0.5 and 3.0 mm. Fluorescence spectra recorded with this instrument are uncorrected.

RESULTS AND DISCUSSION

Spectral Characteristics of Induced Fluorescence: The fluorescence characteristics of α-ecdysone and 20-hydroxyecdysone induced by treatment with H_2SO_4--95% ethanol solutions differed considerably from those of vertebrate steroids such as testosterone, aldosterone, corticosterone, and cortisol. The acid-induced fluorescence of the vertebrate steroids is generally above 500 nm; the acid-induced fluorescence of the ecdyones (Figure 2) is within the range of 400-450 nm (14, 15, 16). The difference in the emission characteristics between the vertebrate and invertebrate hormones undoubtedly has much to do with the α, β -unsaturated ketone group within these steroid ring systems. Robbins et al. (3) pointed out that a major distinction between the ecdysones and many of the vertebrate steroid hormones is that ecdysones have a 7-ene-6-one system in the B ring whereas the vertebrate steroid hormones have a 4-ene-3-one system in the A ring.
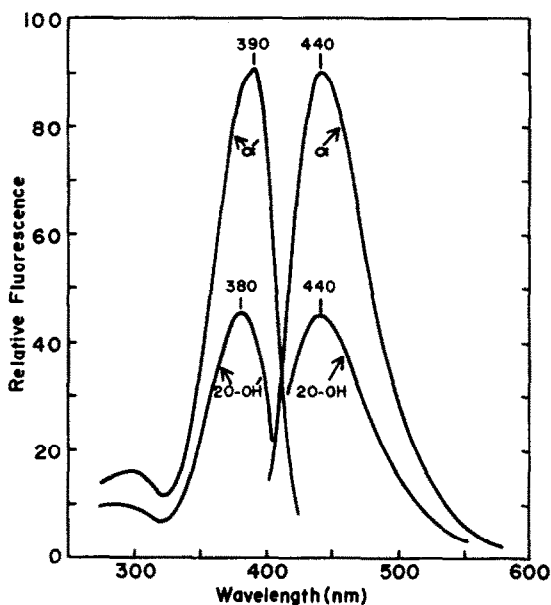
Fig. 2. Acid-induced fluorescence spectra of α-ecdysone and 20-hydroxyecdysone. Fluorescence was induced by treatment of 10 μg samples of α-ecdysone and 20-hydroxyecdysone with 3 ml of H_2SO_4-ethanol solution (3/1, v/v) as described in Methods. Excitation spectra (α′, 20-OH′) were obtained by fixing the emission monochromator at 440 nm. Emission spectra of α-ecdysone and 20-hydroxyecdysone were obtained by fixing the excitation monochromator at 390 and 380 nm, respectively.

The intensity of fluorescence of the ecdysones appears to be dramatically influenced by the positioning of the hydroxyl groups. It has been previously observed that the oxygen-containing groups in a steroid molecule can act as a "fluorescence chromophore" (17). Figure 2 shows that for equivalent amounts (3.3 μg/ml or ca. 7 μM) of α-ecdysone and 20-hydroxyecdysone, the fluorescence intensity of α-ecdysone is approximately twice that of 20-hydroxyecdysone. This response is consistent over a broad range of concentrations as shown in Figure 3.
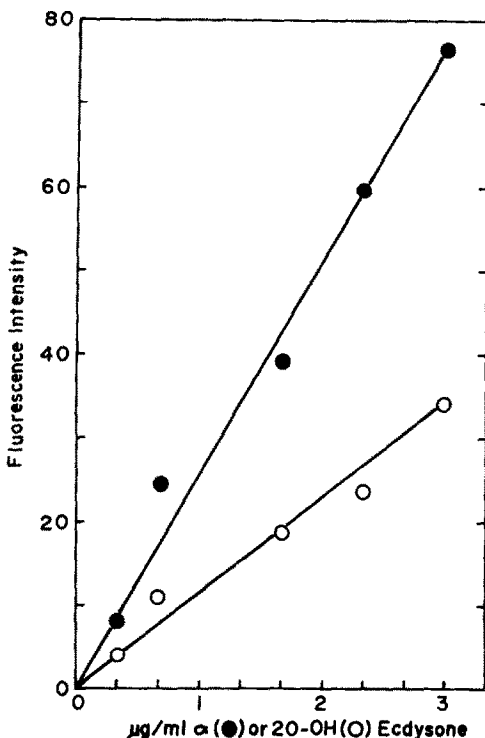
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Fig. 3. Typical fluorescence standard curve of α-ecdysone and 20-hydroxyecdysone treated with H_2SO_4-ethanol solutions (3/1, v/v). Conditions are stated in Methods.

We first thought that the fewer number of hydroxyl groups in α-ecdysone (5 hydroxyls vs. 6 for the 20-hydroxyecdysone) might be the reason for the higher intensity of fluorescence. This theory was therefore tested by comparing the acid-induced fluorescence of $2\beta,3\beta,14\alpha$-trihydroxy-5-cholest-7-en-6-one (triol) with that of α- and 20-hydroxyecdysone. The results showed that the fluorescence intensity of the triol was slightly lower than that of 20-hydroxyecdysone. Therefore, there must be

something unique about the arrangement and/or number of hydroxyl groups in the α-ecdysone molecule.

Other Factors Influencing Ecdysone Fluorescence: The acid-induced fluorescence of steroid molecules has been shown to be affected by such factors as the temperature at which the reaction is carried out, the concentration of acid, the reaction time, and the organic solvent (14, 15). Because many of these factors have been examined for their influence on 20-hydroxyecdysone fluorescence only (14) and because there is a difference in the fluorescence yield between α- and 20-hydroxyecdysone, we felt it necessary to re-examine some of them for their effect on α-ecdysone.

Since the concentration of H_2SO_4 in 95% ethanol was the major factor inducing fluorescence, ecdysone samples (10 μg) were placed in test tubes and the various H_2SO_4-ethanol solutions (3 ml) were added to them. These were stirred and mixed before reading the fluorescence as described in Methods. Obviously, strong acid solutions (ca. 75% in ethanol) are needed to get a full fluorescence response from the ecdysone samples (Figure 4). However, we found that ecdysone fluorescence was stable for up to 30 min. in the 75% H_2SO_4-ethanol solution (cf. 14).

The emission wavelengths of the ecdysones were unaffected by the changes in concentration in the acid-ethanol solutions. However, small hypsochromic shifts in the excitation maxima were observed with decreasing concentration of H_2SO_4. For example, a 10 nm shift in the excitation maximum for α-ecdysone was recorded for the 75 and 33% H_2SO_4-ethanol solutions. At identical conditions, a smaller shift (5 nm) was recorded for 20-hydroxyecdysone.
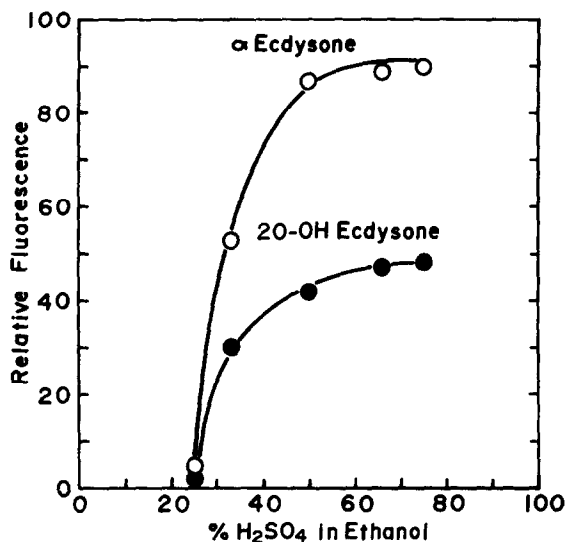
Fig. 4. The effect of H_2SO_4 concentration in ethanol on the fluorescence of 10 μg samples of α-ecdysone and 20-hydroxy-ecdysone. All other conditions are described in Methods.

TLC and Induced Fluorescence of Ecdysones: We found that by spraying standard samples of ecdysones spotted on TLC plates with H_2SO_4 solutions (water or ethanol) we could induce fluorescence _in situ_. However, the fluorescence was extremely variable when read with a TLC scanner and also appeared to be time-dependent, possibly because the acid reacted incompletely with the samples and also charred the samples. Heating the TLC plates at various intervals (0-10 min) at 100° C in an oven increased the sensitivity optimally at about 3 min (by visual observation under long-wave UV light). However, the increased sensitivity made it difficult to quantitate the samples because tailing became a problem. All of these factors detracted from the use of the _in situ_ induced fluorescence of ecdysones as an analytical technique.

An alternative method was to use TLC as a fractionation procedure only. Ecdysone standards were spotted on TLC plates and developed as described.   The   ecdysone-containing   areas   were   delineated   under short-wave UV light by the quenching of the fluorescence background of the TLC plate.   These areas were scraped off, extracted with methanol, and treated with H_2SO_4-95% ethanol (3/1, v/v) as described.   Although the typical ecdysone spectra were observed (Figure 2), the recoveries were extremely variable, which made it difficult to use this method as an analytical technique.

Fluorescence-Quenching: In situ quantitation of UV-absorbing compounds on fluorescent TLC plates can be accomplished by measurements based on the amount of fluorescence-quenching (18). Figure 5 shows that when ecdysone standards were applied to fluorescent TLC plates the amount of quenching was directly proportional to ecdysone concentration between 0.5 and 3 μg. Also, experiments with the triol showed that it responded similarly (unreported results).   However, above 3 μg   of ecdysones, this relationship falls off as the fluorophore-impregnated silica gel becomes saturated with ecdysone.

The fluorescence-quenching method was compared with the HPLC method routinely used in this laboratory. Samples of α- and 20-hydroxyecdysone obtained from incubations of α-ecdysone with α-ecdysone 20-hydroxylase were used as the test samples.   These tests (Table 1) showed that fluorescence-quenching will produce analytical results comparable to those obtained by HPLC measurements. Also, they indicate that extracts of biological material can be analyzed with this method.
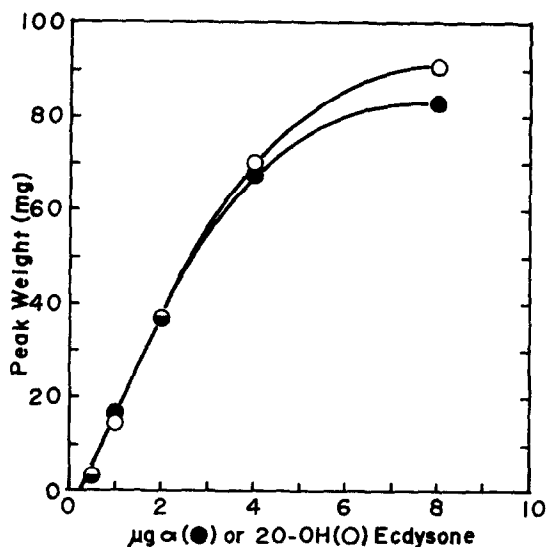
Fig. 5. Typical standard curve for the fluorescence-quenching by α-ecdysone (●) and 20-hydroxyecdysone (O) on fluorescent TLC plates.

Table 1. Analysis of α-ecdysone 20-hydroxylase activity by HPLC and fluorescence-quenching techniques.

	Percent α-ecdysone converted to 20-hydroxyecdysone	
Samples	HPLC	Fluorescence-Quenching
1	18.5	17.7
2	15.0	15.9
3	21.5	20.5
4	24.2	21.3

The major disadvantages of the method are its relatively narrow linear range and the fact that its lower limit of detection is 0.5 μg of ecdysone. The major advantage is that large numbers of samples can be analyzed more rapidly than by conventional HPLC techniques, which makes it a suitable technique for enzyme purification studies. In our

laboratory we routinely analyze 12 to 15 samples a day by HPLC; this

number can be increased 2 to 3 times by the TLC method described herein.

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